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Running Head: MHK in alcohol-induced liver damage

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Abstract

Background: Alcoholic liver disease (ALD) is a leading cause of liver cirrhosis, liver cancer and related mortality. The endocannabinoid system contributes to the development of chronic liver diseases, where cannabinoid receptor 2 (CB2) has been shown to have a protecting role. Thus, here we investigated how CB2 agonism by 4'-O-methylhonokiol (MHK), a biphenyl from *Magnolia grandiflora*, affects chronic alcohol-induced liver fibrosis and damage in mice.

Methods: A combination of alcohol (10% vol/vol) and CCl4 (1ml/kg) was applied to C57Bl/6 mice for 5 weeks. MHK (5mg/kg) was administered daily. Liver damage was assessed by serum AST and ALT levels, histology, gene and protein expression. Endocannabinoids (ECs) and related lipid-derivatives were measured by liquid chromatography and mass spectrometry (LC-MS) in liver tissues. In vitro, MHK was studied in TGFβ1-activated hepatic stellate cells (HSC).

Results: MHK treatment alleviated hepatic fibrosis, paralleled by induced expression of matrix metalloproteinases (MMP) -2, -3, -9 and -13, and downregulation of CB1 mRNA. Necrotic lesions and hepatic inflammation were moderately improved, while IL-10 mRNA increased and IFNγ, Mcl-1, JNK1 and RIPK1 normalized by MHK. Hepatic levels of anandamide (AEA) and related N-acetylethanolamines (NAEs) were elevated in MHK group, whereas fatty acid synthase and diacylglycerol O-acyltransferase 2 expression reduced. In vitro, MHK prevented HSC activation and induced apoptosis via induction of bak1 and bcl-2.

Conclusions: MHK revealed hepatoprotective effects during alcohol-induced liver damage through the induction of MMPs, AEA and NAEs, and prevention of HSC activation, indicating MHK as a potent therapeutic for liver fibrosis and ALD.

Keywords: alcohol, liver, cannabinoids, methylhonokiol, therapy

INTRODUCTION

Excess alcohol consumption is the most frequent cause of liver cirrhosis worldwide, and in 2010, alcoholic liver cirrhosis was responsible for 493,300 deaths (47.9% of all liver cirrhosis deaths), representing 0.9% of all deaths regardless the cause (1). Moreover, alcoholic cirrhosis is the second most common indication for liver transplantation, accounting for approximately 40% of all primary liver transplants in Europe and about 25% in the United States (2). Despite this huge burden of disease, no effective pharmacological treatment for alcoholic liver disease (ALD) is approved, and drug interventions are primarily focused on achieving abstinence or risk reduction by decreasing alcohol consumption (3). Hence, novel therapeutic targets are clearly needed to specifically treat established ALD.

Mounting evidence indicate that the endocannabinoid system (ECS) plays an important role in various liver diseases including viral hepatitis, non-alcoholic fatty liver disease (NAFLD), ALD, hepatic encephalopathy and autoimmune hepatitis (4). In particular, upregulation of cannabinoid receptors (CB) 1 and 2 in mostly all cronic liver diseases, cirrhosis and related disturbances were convincingly demonstrated, leading to the intriguing therapeutic concept of functional antagonism of these receptors in liver pathophysiology, with CB1 promoting and CB2 protecting from liver damage (5, 6) (7). Thus, in *CB1*$^{-/-}$ and *CB2*$^{-/-}$ mice, deletion of CB1 improved hepatic fibrosis and steatosis induced by carbon tetrachloride or high-fat-diet, whereas the lack of CB2 increased collagen deposition, liver fat and inflammation (8, 9). In experimental alcoholic models the CB2 agonist JWH-133 showed protective effects, as reflected by improved hepatic fibrosis, steatosis, inflammation and liver regeneration (10-12). Similarly, another CB2 agonist HU-308 alleviated tissue injury, decreased inflammatory cell infiltration and apoptosis during ischemia reperfusion (12).

As a traditional medicine, *Magnolia* extracts has been used to treat gastrointestinal, anxiety and allergic diseases in Asian countries for several centuries (13-15). Honokiol, magnolol,

and 4'-O-methylhonokiol (MHK) are considered the major bioactive constituents of *Magnolia grandiflora L.* Although the structural similarity, MHK is the only compound which showed significant bioactive properties on the ECS as a CB2 receptor agonist and COX-2 substrate specific inhibitor for EC oxygenation (16-18). Recent studies revealed that magnolol and honokiol positively affect body fat accumulation and insulin resistance in high-fat diet (HFD)-fed mice (19, 20), as well as improved fasting and plasma insulin levels in a type 2 diabetic model, alleviated hepatic oxidative damage, hyperglycemia and hyperlipemia (21-23). Also, honokiol was found to induce apoptotic death in activated rat HSCs suggesting an antifibrotic effect, while there was no such effect on hepatocytes (24). *Magnolia officinalis* extracts showed beneficial effects on hepatic lipid accumulation, inflammation, oxidative stress, and apoptosis in a HFD-induced obese mouse model (25-27). *M. officinalis* treatment of the rats on alcohol diet completely normalized serum liver enzyme levels, reversed steatosis, and completely inhibited the increased maturation of sterol regulatory element-binding protein-1c (SREBP1c) in the liver (28). Thus, *Magnolia* extracts seem to be promising candidates for ALD therapy. Here, we studied the therapeutic potential of MHK, a pure bioactive component of *M. grandiflora* and *M. virginiana,* in a murine model of alcohol-induced liver damage and accompanying fibrosis.

MATERIAL AND METHODS

Materials

The procedure of isolation and purification of 4'-O-Methylhonokiol is described in details by A. Chicca et al. in (18).

Animals

Male C57Bl/6 mice, 10 weeks age (20-25g) were obtained from Charles River, Sulzfeld, Germany, and kept under a 12h light–dark cycle with free access to rat chow (Provimi Kliba AG, Kaiseraugst, Switzerland) and water *ad libitum*. Animal experiment and all experimental protocols were approved by the research animal ethics committee and by the local board on animal care and experimentation of Canton Bern and were performed according to international guidelines concerning the conduct of animal experimentation.

Cells

Human hepatic stellate cells (HSC; ScienCell Research Laboratories, Carlsbad, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 U/mL streptomycin. Cells were maintained at 37 °C in 5% CO2 humidified atmosphere. Cells were seeded onto 12-well plates and after reaching semi-confluent state starved in serum-free medium for next 24h. Treatment with 1-10µM MHK was performed for 6–24h in a complete serum-free medium.

In vivo model of liver injury and treatment

Liver fibrosis was induced in male adult C57Bl6 mice by chronic administration of combination of 10% (vol/vol) alcohol (EtOH) in sweetened drinking water (5% sucrose) daily and CCl4 (1ml/kg, 1:10 in corn oil), by intraperitoneal injections (i.p.), 2x weekly. This

model replicates the "multiple hits" model, where CCl4 is used as an additional trigger to enhance the effect of ethanol on fibrosis development (29), lacking in other animal models, such as Lieber-deCarli, Tsukamoto-French or NIAAA (30, 31). Therefore, individual control groups (ethanol alone, CCl4 alone) were not used. The adaptation to alcohol included gradual increase and application of EtOH in drinking water starting from the low dose 1% at day 1 to maximum dose 10% at day 7. Therafter, animals were divided into 3 groups: 1. sham (sweetened drinking water) + vehicle (n=4); 2. EtOH + CCl4 + vehicle (n=8); 3. EtOH + CCl4 + MHK 5mg/kg/day (n=6). MHK was given by i.p. daily, for 5 weeks. As the effects of MHK on untreated animals on normal chow diet have been tested in (20), this control group was not included. At the end of the experiment (2 days after the last CCl4 injection) animals were sacrificed under isofluorane anesthesia by puncture of the right heart ventricle and exsanguination. Blood and liver tissue samples were either snap frozen or collected in formalin for further analyzes.

Standard serum parameters

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in the internal laboratory of University Hospital Zürich, Institute of Clinical Chemistry.

Liver histology, immunohistochemistry and morphometry

Tissue samples were fixed in formalin and embedded in paraffin using routine histological procedures. Deparaffinized 4µm liver sections were stained with hematoxylin and eosin (H&E), and Sirius Red, according to the standard protocols and manufacturers' recommendations. Alpha smooth muscle actine (αSMA) stain was performed using anti-αSMA rabbit polyclonal antibody (Abcam; 1:200, overnight, +4°C) and detection performed with DAB Peroxidase (HRP) Substrate Kit (Dako, Denmark).

Morphometric quantification of Sirius Red staining was performed using an ImageJ 1.47t software system (32), and the results are presented as an area count, analysed from 5 fields per section (magnification 40x). Hepatic inflammation was evaluated semiquantitatively, based on the distribution of the inflammatory cells (1-4, single cells to multiple cells/islands/mild semi-confluent; 5-7, mild (semi-) confluent - moderate confluent; 8-10 severe confluent). Hepatic necrosis was evaluated semiquantitatively, based on the following scoring system: 1 – single necrotic cells; 2 – semi-confluent (not connected bridges of necrotic cells between portal and/or central areas); 3 – confluent (connected bridges of necrotic cells between portal and/or central areas). All histological analyses were performed in blinded fashion.

TaqMan PCR

Total RNA was isolated from 30mg of liver tissue using the RNeasy kit (Qiagen, Basel, Switzerland), and cDNA was transcribed from 1µg of RNA using M-MLV reverse transcriptase (Invitrogen, Basel, Switzerland) with a random hexanucleotide mix (Roche, Basel, Switzerland). Quantitative real-time PCR was carried out on an ABI 7700 sequence detector (Applied Biosystems, Rotkreuz, Switzerland). All TaqMan probe and primer sets were obtained as ready-to-use kits from Applied Biosystems (Rotkreuz, Switzerland). For normalization the housekeeping gene GAPDH was amplified in a parallel reaction.

Western blot analysis

All devices, materials and reagents for western blotting were obtained from Thermo Fischer Scientific, Reinach, Switzerland, if not stated otherwise. Liver tissue protein extracts were prepared using T-PER® (Tissue Protein Extraction Reagent) containing Complete™ protease inhibitor cocktail, Sample Reducing Agent Bolt™ (10x), and LDS Sample Buffer Bolt™

(4x), according to manufacturers' recommendations. 10µl (20µg) of lysates were applied to Bolt™ 4-12% or 12% Bis-Tris Plus gels transferred to iBlot™ 2 NC Regular Stacks using iBlot® 2 Dry Blotting System and stained with 0,5% Ponceau S to assure equal protein loading and transfer. Membranes were blocked and incubated with the primary antibody overnight at +4°C, incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (**Supplementary Table 1**), and proteins detected using the WesternBright™ Sirius western blotting detection kit (Advansta, CA, USA). For quantification analysis, all proteins of interest were normalized to GAPDH.

Viability assay

Cell viability was measured using Quick Cell Proliferation Assay Kit II (ab65475) (Abcam, Cambridge, UK), according to manufacturer's recommendations.

Quantification of ECs and related lipids with LC-MS/MS

ECs, N-acetylethanolamines and arachidonic acid were measured in the liver by liquid chromatography tandem mass spectrometry (LC-MS), as described in Supporting Information.

Statistics

Comparison was performed with the one-way ANOVA Kruskal-Wallis test, or Mann-Whitney U test, and survival was quantified with Kaplan-Meier analysis using the Graph Pad statistics software (Graph Pad Software Inc., San Diego, CA, USA). Results are given as means ± SD and considered statistically significant with a p-value <0.05.

196 **RESULTS**

197 ***MHK improves hepatic fibrosis***

198 Five weeks of EtOH/CCl4 administration caused approximately 30% mortality, whereas
199 MHK administration improved animal survival (**Fig. 1A**). Body, liver and spleen weights
200 remained mainly unchanged after EtOH/CCl4 and MHK administration (not shown).
201 Liver histology of fibrotic mice showed mild to moderate fibrosis with wide-spread
202 proliferation of αSMA-positive portal myofibroblasts and HSC aligning fibrotic septas and
203 necroinflammation areas (**Fig. 1B**). Morphometric analysis of Sirius Red staining
204 demonstrated less pronounced extracellular matrix deposition after MHK treatment (**Fig. 1C**),
205 as well as actin protein expression was similar to that in untreated healthy control mice (**Fig.**
206 **1D**). Though no major changes were noticed at mRNA levels for PCα1, αSMA and TGFβ1
207 by MHK, strongly upregulated MMP-2, -3, -9 and -13 levels were detected (**Fig. 1E**).
208
209 ***MHK exerts mild anti-inflammatory effects in the liver***
210 Toxic injury in mice resulted in pronounced perilobular neutrophilic infiltration, aligning
211 fibrotic septas and necrotic areas (**Fig. 2A**). Semi-quantitative evaluation revealed a reduced
212 infiltration of immune cells upon MHK treatment (**Fig. 2B**), although at mRNA levels no
213 major changes were detected, except of dramatic restoration of IFNγ. Although not
214 statistically significant, TNFα mRNA was slighlty upregulated by MHK, whereas anti-
215 inflammatory IL-10 increased too (**Fig. 2C**).
216
217 ***MHK protects from hepatocytes injury***
218 Administration of EtOH/CCl4 for 5 weeks resulted in moderate hepatotoxic injury, as
219 evidenced by single cell decay or confluent areas of necrotic lesions from portal to central
220 veins, or portal to portal areas (**Fig. 3A**). Semi-quantitative evaluation of necrotic areas

221 indicated that MHK seems to have a protective effect from toxic damage of alcohol and
222 CCl4, although this did not reach statistical significance (**Fig. 3A**). However, serum liver
223 enzymes and markers of liver damage ALT and AST were markedly elevated in alcoholic
224 control group, and MHK significantly improved AST levels almost by 50% (**Fig. 3B**).
225 Apoptosis-regulating bax, bak and bcl-xl proteins were not affected strongly neither in
226 alcohol controls, nor by MHK treatment, however, a significant reduction of Mcl-1 and
227 necrosis-regulating JNK1 was observed in the liver tissues from MHK group (**Fig. 3C,D**).
228 Necroptosis-related RIPK1 was significantly downregulated in alcohol control group,
229 whereas MHK normalized its expression back to normal levels (**Fig. 3E**). RIPK3 had similar
230 tendency but not statistically significant.
231 Alcohol-metabolising and oxidative stress producing enzymes CYP2E1, CYP4A14 and p47-
232 phox were not affected by MHK (**Fig. 3F**).
233
234 ***MHK treatment increased hepatic AEA and related acetylethanomalides***
235 The levels of ECs anandamide (AEA) and 2-arachydonoilglycerol (2-AG), N-
236 acetlyethanolamines (NAEs) and arachidonic acid were measured in livers of all
237 experimental groups. MHK treatment significantly increased by approximately 3-times the
238 concentrations of AEA and the NAEs oleoylethanolamide (OEA), palmitoylethanolamide
239 (PEA) and linolenoylethanolamine (LEA) including arachidonic acid (**Fig. 4A**). On the
240 contrary, 2-AG levels were not affected by MHK treatment (**Fig. 4A**).
241 The gene expression of some lipid metabolising enzymes was investigated in order to identify
242 a possible cause for the increase of AEA and NAEs. Thus, MHK upregulated the expression
243 of PPARγ and PNPLA3 mRNA compared with CCl4/EtOH treatment alone, and inhibited at
244 the same time FAS and DGAT2 protein synthesis (**Fig 4B,C**). No major impact on the
245 expression of SREBP1, MBOAT7 and ALDH1A1 was detected (**Fig. 4B,C**).

In parallel, the expression of CB1 and CB2 receptors and the major enzymes involved in AEA and 2-AG biosynthesis and degradation was evaluated. Interestingly, MHK abolished CB1 receptor overexpression, induced by hepatotoxins CCl_4 and alcohol, whereas no statistically significant effects were detected on CB2, FAAH, MAGL, NAPE-PLD, PTPN22, DAGLβ and COX-2 mRNA expression (**Fig. 4D**).

MHK reduced human HSC activation and induced apoptosis

In vitro, human HSC were stimulated by TGFβ1 at 10ng/ml to induce profibrogenic phenotype, and when treated with MHK at 5-10μM, significant downregulation of PCα1(I), TGFβ1, αSMA, PDGFRβ, TNC, and TIMP1 was observed after the treatment for 24h with the highest concentration 10μM (**Fig. 5A**). At protein level, this was confirmed by HSC activation markers tenascin c and actin, which were reduced by MHK 10- and 2-fold, respectively (**Fig. 5B**).

Cell viability was strikingly reduced by MHK at higher doses 10 and 20μM from 25 to 85%, respectively (**Fig. 6A**), paralleled by the 2-3-fold induction of bak1 and bcl2 mRNA at 10μM MHK (**Fig. 6B**).

DISCUSSION

MHK is traditionally used in Chinese medicine for the therapy of depression and anxiety, and has been tested in experimental animal models of memory impairment or Alzheimer's disease (14, 33-36). Although only a few studies investigated the effects of MHK in peripheral disorders, experimental data suggests a potential anti-tumor property of MHK in the treatment of oral squamous cell, prostate and colon carcinoma *in vitro* and *in vivo* (37-39). Other studies reported anti-inflammatory and anti-osteoclastogenic effects for MHK (40). Regarding liver diseases, only one study demonstrated that MHK may positively impact on features of the metabolic syndrome, such as obesity, insulin resistance and non-alcoholic steatohepatitis in a mouse high-fat-diet model (20). Considering the putative role of ECS in chronic liver diseases, we studied the effects of MHK in alcohol-induced liver damage in mice, and showed that it significantly reduces serum alanine and aspartate aminotransferases levels, significantly improves mice survival and alleviates liver fibrosis and damage via modulation of MMP-2, -3 and -13 and CB1 expression. The important role of matrix metalloproteinases degrading different types of collagens and other extracellular matrix proteins in fibrosis development is well established, and the particular anti-fibrotic role of MMP-2, -3 and -13 has been convincingly demonstrated (41). Even though the expression of the several key fibrogenic genes, such as PCα1, TGFβ1 and αSMA mRNA have not been found to be strongly affected by MHK in our animal model, the protein levels of actin were significantly less in MHK group, compared to fibrosis control, reflecting reduced HSC and myofibroblast number or activity. In addition, MMPs expression, activation, and thus, fibrolysis might be sufficient to shift the balance between fibrogenesis and fibrolyis towards fibrosis resolution. In vitro, MHK strikingly suppressed the expression of PCα1(I), actin, TGFβ1, TIMP1, tenascin, PDGFRβ and induced HSC apoptosis, confirming the direct anti-fibrogenic efficacy of MHK on stellate cells and activated fibroblasts, which possibly could

be detected only at certain time points in vivo. The profibrogenic role of CB1 receptor has been repeatedly reported, and numerous investigators showed that genetic deletion or pharmacological antagonism of CB1 improves hepatic fibrosis, inflammation and liver fat accumulation in various experimental models (42). On the contrary, CB2 activation exerts anti-inflammatory and anti-fibrotic effects in liver and other organs (8, 10, 43). Interestingly, in our study, MHK did not affect the expression of CB2 receptor, but strongly abolished the CCl4/EtOH-induced upregulation of CB1, thus potentially leading to the inactivation of one of the major pro-fibrogenic pathways within the ECS network.

Despite many studies providing evidence for a protective effect of MHK from neuroinflammation (13, 33, 36), the effect of MHK treatment on hepatic necroinflammation was only mild. Mice treated with MHK had slightly less neutrophilic infiltration in the periportal and pericentral areas of the liver. There was a trend towards improved levels of IL-6, IFNγ and induced anti-inflammatory IL-10 mRNA by MHK, but these differences failed to reach statistical significance. Previous findings by others indicated that MHK may have an inhibitory effect on COX-2 (18, 44, 45), but again, this was not confirmed in experimental ALD, compatible with our findings of a lack of a striking effect on hepatic inflammation.

When evaluating liver toxicity, we noticed that the premier type of hepatic cell death is necrosis and not apoptosis, as demonstrated by hepatocytes ballooning and disappearing nuclei. Therefore, to more specifically investigate the type of cell death the expression of genes coding for proteins involved in apoptosis, necrosis and necroptosis were evaluated. Although the necrotic lesions in the MHK group seem to be less striking, semiquantitative quantification did not reveal statistically significant difference with a control group. However, the levels of the liver function enzymes AST and ALT in the serum of mice receiving MHK showed a significant improval. In line with this observation, downregulation of necroptotic RIPK1 in alcohol-treated mice was restored by MHK to normal levels. RIPK1

had been shown to have a protective role, as RIPK1-/- mice die at birth, and rip-/- fibroblasts and thymocytes are sensitive to TNFα-induced cell death, as well as hematopoietic RIPK1 deficiency triggers both apoptotic and necroptotic death (46-48). This indicates that MHK either directly or indirectly stimulates RIPK1 signaling pathways pivotal for survival.

No effects of MHK were found in the expression of proapoptotic bak, bax and bcl-xl proteins, whereas Mcl-1 and necrosis-mediating JNK1 were increased by EtOH/CCl4 administration, and markedly decreased to normal levels with MHK treatment. All together, these data point to a protective potential of MHK in alcohol-mediated hepatic injury via the regulation of RIPK1, Mcl-1 and JNK signaling pathways.

Reduction of oxidative damage by MHK in embryopathies induced by nicotine, as well as improved memory impairment by the same mechanism had been reported previously (34, 49-51), and might partially explain the improvement of alcohol-induced liver damage in our study, which is also mediated by oxidative stress and free radical formation from alcohol-induced cytochrome P450 2E1 (CYP2E1). However, the expression of CYP2E1, CYP4A14 and p47-phox was not affected by MHK, indicating that MHK either influences other downstream proteins, or has a direct neutralizing effect on alcohol, CCl4 and related oxidative stress products.

In livers from mice treated with MHK, levels of AEA and NAEs were increased by approximately three times compared with CCl4/EtOH, while 2-AG concentration was not affected. This difference seems to be associated with the distinc modulation of the expression of AEA and 2-AG degrading enzymes. MHK showed a clear trend towards lowering the expression of the main AEA and NAEs hydrolytic enzyme FAAH (although not being statistically significant), while it did not affect the 2-AG hydrolytic enzyme MAGL. None of the AEA, NAEs and 2-AG biosynthetic enzymes were modulated by MHK. This data suggest that the specific increase of AEA and NAEs levels over 2-AG may result, at least in part,

from a reduced degradation. AEA and NAEs are PPARs ligand and can potentially contribute to the MHK protective effect. In addition, reduced AEA hydrolysis limits the release of free arachidonic acid which is the main COX-2 substrate, thus ultimately leading to reduced formation of pro-inflammatory prostaglandins.

To elucidate whether other lipid biosynthesic or degradating pathways were involved in AEA and NAEs increase, also keeping in mind potential pro-steatotic effects of CB2 activation, we measured the expression of genes coding for related enzymes and transcription factors pertinent for lipid turnover. We found that PPARγ and PNPLA3 mRNA expression was restored or induced by MHK, whereas SREBP1 and MBOAT7 were not affected neither by EtOH/CCl4 nor by MHK. This suggests a preponderance of lipid hydrolysis over synthesis, as also reflected by the downregulation of fatty acid synthase and diacylglycerol O-acyltransferase 2 by MHK treatment in our model. In line with this observation, MHK was identified as a PPARγ agonist (38, 52), delivering an explanation for its potential anti-steatotic functions, possibly via the insulin sensitizing and lipid lowering effects of PPARγ (20, 53).

The relatively mild molecular effects of MHK in our model could be explained by the rapid degradation of MHK, as shown *in vitro* using rat liver microsomes and cytosol by Lee and coworkers, who demonstrated a high systemic plasma clearance and large volume of distribution, with an oral dose of 10 mg/kg resulting in a peak plasma concentration of only 24.1±3.3 ng/ml at 3h and a low estimated bioavailability (54).

Our study has some weaknesses related to the animal model used which produces only an incomplete pattern of ALD as encountered in humans. Rodents are notoriously resistant to the hepatotoxic effects of alcohol due to species-related differences, and rats or mice only develop fibrosis when exposed to alcohol in combination with a second toxin (e.g. carbon tetrachloride, thioacetamide) or major dietary manipulations (e.g. choline/methionine

deficiency) (31). In particular, our model produces little steatohepatitis, and lacks typical microscopic lesions such as Mallory-Denk bodies. However, our data demonstrate a protective effect of MHK on liver damage mediated by alcohol administration, resulting in reduced mortality, alleviated fibrosis and necroinflammation, possibly via direct antifibrotic effects and modulation of lipid metabolism. Though we expect similar effects in other models of alcohol-induced liver damage, further studies are necessary to understand whether the protective effects of MHK depend mainly on one pharmacological property (e.g. CB2 activation) or are the consequence of polypharmacoloigcal actions in the ECS (e.g. CB2 activation, limiting CB1 expression, increasing AEA and NAEs levels).

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Conflict of interest

The authors declare that they have no actual or potential competing financial interests.

Author contribution

EP - performed most of the experiments and analyses, wrote the paper, retrieved the funding for the study

AC - performed the ECs measurements and LC-MS/MS analyses, critically revised the paper

VP - performed the ECs measurements by LC-MS/MS, critically revised the paper

SM - executed the animal experiment, critically revised the paper

AdG - conceived the animal experiment, supervised its execution, critically revised the paper

JG - conceived the animal experiment, supervised its execution, critically revised the paper

NS - conceived the animal experiment, supervised its execution, critically revised the paper

FS - conceived the experiment, supervised its execution, wrote the paper

All authors contributed to the paper draft and approved its final version prior to submission.

ABBREVIATIONS LIST

AEA – N-arachidonoylethanolamine (anandamide)

ALD – alcoholic liver disease

ALDH1A1 - aldehyde dehydrogenase 1 family member A1

ALT – alanine aminotransferase

AST – aspart ataminotransferase

2-AG – 2-Arachidonoylglycerol

αSMA – alpha smooth muscle actin

CB – cannabinoid receptor

CCl4 – carbon tetrachloride 4

CYP – cytochrome

DGAT2 – diacylglycerol O-acyltransferase 2

EC - endocannabinoid

ECS – endocannabinoid system

FAS – fatty acid synthase

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

HFD – high-fat diet

HSC – hepatic stellate cell

IFNγ – interferon gamma

IL – interleukin

JNK – c-Jun N-terminal kinase 1

LC-MS – liquid chromatography and mass spectrometry

LEA – linolenoylethanolamine

MBOAT7 - membrane bound O-acyltransferase domain containing 7

Mcl-1 – myeloid leukemia cell differentiation protein – 1

MHK – methylhonokiol

MMP – matrix metalloproteinase

NAFLD – non-alcoholic fatty liver disease

NAE - N-acetylethanolamine

NE – noladin ether

OEA – oleoylethanolamine

PCα1 – procollagen alpha 1 (I)

PEA – palmitoylethanolamide

PPARγ – peroxisome proliferator-activated receptor gamma

PNPLA3 – patatin-like phospholipase domain-containing protein 3

RIPK – receptor interacting serine/threonine kinase

SREBP1 – sterol regulatory element-binding protein-1

TGFβ1 – transforming growth factor beta 1

TNFα – tumor necrosis growth factor alpha

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599 **FIGURE LEGENDS**

600 **Figure 1. Survival, morphology and hepatic fibrosis after MHK treatment**

601 (**A**) Mice survival after 5 weeks of EtOH/CCl4 and MHK administration; (**B**) Representative
602 liver sections of EtOH/CCl4 and EtOH/CCl4 + MHK (Sirius Red and αSMA IHC,
603 magnification 10x). Moderate deposition of collagen fibers can be noticed in livers from
604 fibrosis control group, whereas with MHK the severity of fibrotic matrix deposition is less
605 pronounced. Strong positivity for αSMA differentiates activated myofibroblasts and HSC
606 aligning fibrotic septas (brown); (**C**) Morphometric quantification of Sirius Red staining by
607 ImageJ software from all experimental groups; (**D**) Actin protein expression levels measured
608 by Western Blot and normalized to GAPDH; (**E**) Fibrosis-related mRNA expression,
609 measured by TaqMan PCR and normalized to GAPDH (mean ± SD; *p<0.05, ns – non
610 significant).

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612 **Figure 2. MHK effect on hepatic inflammation**

613 (**A**) H&E staining and (**B**) semiquantitative evaluation of hepatic inflammation from
614 EtOH/CCl4 and MHK groups. Mild to massive neutrophil infiltration can be noticed around
615 necrotizing portal and central ares of the liver. (**C**) Inflammation-related mRNA expression,
616 measured by TaqMan PCR and normalized to GAPDH (mean ± SD; *p<0.05, ns – non
617 significant).

618

619 **Figure 3. MHK effect on hepatic necrosis**

620 (**A**) Representative example of typical necrotic lesions from H&E staining and semi-
621 quantitative analysis of necrotic areas, based on the severity and distribution of the necrotic
622 cells. Aggregates of swelling hepatocytes with disappeared or empty nuclei and lost stain,
623 indicating predominantly necrotic/necroptotic type of cell death vs apoptotic is clearly visible

on the section (yellow arrow, magnification 40x). (**B**) Serum liver enzymes ALT and AST (U/L) (mean ± SD; *p<0.05, ns – non significant). (**C, D**) Western blotting and corresponding quantification of apoptosis-regulating proteins bak, bax, bcl-xl, Mcl-1 and JNK1 using ImageJ software system. (**E**) Necroptosis-regulating kinases RIPK1 and RIPK3 mRNA, measured by TaqMan PCR and normalized to GAPDH (mean ± SD; *p<0.05, ns – non significant). (**F**) Alcohol-metabolizing and oxidative stress-related proteins measured by Western Blot and TaqMan PCR, normalized to GAPDH (mean ± SD; *p<0.05, ns – non significant).

Figure 4. MHK effect on hepatic lipid metabolism

(**A**) AEA, 2-AG OEA, PEA, LEA and arachidonic acid (AA) levels in the liver (mean ± SD; ***p<0.001, **p<0.01, *p<0.05). (**B**) PPARγ, SREBP1, PNPLA3 and MBOAT7 mRNA, measured by TaqMan PCR, normalized to GAPDH (mean ± SD; *p<0.05, ns – non significant). (**C**) Western blot and corresponding quantification of the bands of enzymes involved in lipid synthesis FAS, DGAT-2 and ALDH1A1 using ImageJ software system (mean ± SD; *p<0.05, ns – non significant). (**D**) ECS-related mRNA expression: cannabinoid receptors CB1 amd CB2, ECs degrading and metabolizing enzymes FAAH, MAGL and COX-2, ECs synthesizing enzymes NAPE-PLD, PTPN22 and DAGLβ, measured by TaqMan PCR and normalized to GAPDH (mean ± SD; *p<0.05, ns – non significant).

Figure 5. MHK inhibited HSC activation

(**A**) PCα1(I), αSMA, TGFβ1, PDGFRβ, TNC, IL-6, TIMP1, MMP-3 and MMP-13 mRNA expression in HSC activated by 10ng/ml TGFβ1, measured after 24h by TaqMan PCR and normalized to GAPDH (mean ± SD; *p<0.05, ns – non significant); (**B**) actin and tenascin protein expression in HSC activated by 10ng/ml TGFβ1, measured after 24h by Western

649 Blot, analyzed by ImageJ and normalized to GAPDH (mean ± SD; *p<0.05, ns – non
650 significant).

651 **Figure 6. MHK induced HSC apoptosis**

652 (**A**) HSC viability measured by WST assay after 24h of treatment with 10ng/ml TGFb1 and
653 1-20μM MHK; (**B**) bak1, bax, Mcl-1, bcl2 and bcl-xl mRNA expression measured after 24h
654 by TaqMan PCR and normalized to GAPDH (mean ± SD; *p<0.05, ns – non significant).

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